Characterization and Pathogenesis of Aerosolized Eastern Equine Encephalitis in the Common Marmoset (*Callithrix jacchus*)

Aimee I. Porter[1]*, Rebecca A. Erwin-Cohen[1]*, Nancy Twenhafel[2], Taylor Chance[2], Steven B. Yee[3], Steven J. Kern[4], David Norwood[5], Laurie J. Hartman[5], Michael D. Parker[1], Pamela J. Glass[1], Luis DaSilva[3#]

*Equally-contributing authors
[#]Corresponding author, luis.c.dasilva2.ctr@mail.mil Tel: 301-619-1028

Affiliations:

[1]United States Army Medical Research Institute of Infectious Diseases (USAMRIID), Virology Division; [2]Pathology Division; [3]Center for Aerobiological Sciences; [4]Research Support Division; [5]Diagnostic Systems Division, Frederick, MD 21702

Abstract

Licensed antiviral therapeutics and vaccines to protect against eastern equine encephalitis virus (EEEV) in humans currently do not exist. Animal models which faithfully recapitulate the epidemiology of the human EEEV disease are needed to satisfy requirements of the Food and Drug Administration (FDA) for clinical product licensing under the Animal Rule. In an effort to meet this requirement, we estimated the median lethal dose and described the pathogenesis of aerosolized EEEV in the common marmoset (*Callithrix jacchus*). Five marmosets were exposed to aerosolized EEEV FL93-939 in doses ranging from 2.4×10^1 PFU to 7.95×10^5 PFU, with a median lethal dose of 2.05×10^2 PFU. Euthanasia criteria was met by day 4 post exposure in the highest dose marmoset but animals at lower inhaled doses had a protracted disease course where euthanasia criteria was not met until as late as day 19 post exposure. Clinical signs were observed as early as 3 to 4 days post-exposure, including fever, ruffled fur, decreased grooming, and lymphopenia. Clinical signs of disease increased in severity as disease progressed to include decreased body weight, subdued behavior, tremors, and lack of balance. Fever was evident as early as day 2-3 post exposure in the highest dose groups and hypothermia was observed in several cases as animals became moribund. Infectious virus was found in several key tissues, including brain, liver, kidney, and lymph nodes. Clinical hematology results included early neutrophilia, lymphopenia, and thrombocytopenia. Key pathological changes included meningoencephalitis and retinitis. Immunohistochemical staining for viral antigen was positive in the brain, retina, and lymph nodes. More intense and widespread IHC labeling occurred with increased aerosol dose. In summary, we have estimated the medial lethal dose of aerosolized EEEV and described the pathology of clinical disease in the marmoset model. The results

demonstrate that the marmoset is an animal model suitable for emulation of human EEEV disease in the development of medical countermeasures.

Key words

Animal model, alphavirus, immunity, nonhuman primate, pathogenesis

Introduction

New world alphaviruses, such as EEEV, are the cause of highly pathogenic disease in equines and humans and which can manifest as severe encephalitis in humans. Due to high infectivity, ability to induce devastating disease, ease of production, high degree of stability, and the potential for aerosolization, EEEV is considered a potential biological threat agent against both the Warfighter and civilians and is classified as a category B pathogen by the CDC and NIAID (NIAID, 2016). EEEV is a single-stranded, positive-sense RNA virus found in the eastern half of North America (Reed, 2007). EEEV is considered one of the deadliest mosquito-borne alphaviruses with mortality rates as high as 80-90% in horses (Go, 2014). Long term neurological sequelae are observed in about 66% of surviving equines (Go, 2014). In humans, severe infection can result in neurological invasion and encephalitis with mortality rates ranging from 33-70% (Gaensbauer, 2014; Aréchiga-Ceballos & Aguilar-Setién, 2015). Encephalitic patients often experience severe symptoms of disease including high fever, headache, vomiting, general or focal seizures, and coma; long term neurological sequelae may persist in survivors and include both motor and cognitive impairments (Petersen & Gubler, 2003; Aréchiga-Ceballos & Aguilar-Setién, 2015). Until recently, EEEV was considered to consist of four genetic lineages (Arrigo, 2010). Lineage I was considered to be the North American (NA) variant of EEEV, while lineages II, III and IV were the South American (SA) variants. The latter three lineages have now been classified as a new viral species, Madariaga virus (Arrigo 2010). EEEV strains (previously referred to as NA strains) are transmitted by the mosquito vector and are prevalent in coastal and swampy regions of the eastern United States; between 2003 through 2012, 89 cases of EEEV were confirmed in the US (Gaensbauer, 2014). The geographical range of EEEV infections stretched from the Gulf to Atlantic coasts, and the vast majority of cases reported were

from Florida, Massachusetts, Alabama, North Carolina, New Hampshire, Louisiana, and Georgia (Gaensbauer, 2014).

The need for licensed vaccines and antiviral therapeutics for human use in the event of EEEV infection has fostered research efforts to characterize animal models which can be used to assess efficacy of novel countermeasures. Marmosets are a nonhuman primate (NHP) species that have been used in a wide range of research efforts for human disease such as reproductive biology, behavioral research, and most importantly biomedical research (Zühlke, 2003; Garea-Rodriguez, 2016). Marmosets have previously been used to assess intranasal EEEV infection (Adams, 2008). The small size of the marmoset allows for easy handling, reasonable housing space, and provides greater amounts of test material for research than traditional rodent models. The ease of breeding in captivity coupled with the fact that the numbers of animals in the wild are not threatened represent additional considerations for the justification and use of this species in biomedical research (Mansfield, 2003, Zühlke, 2003, Adams, 2008).

In the present study, the aerosol route of EEEV infection was evaluated in the common marmoset. Marmosets were exposed to a range of aerosolized EEEV doses to estimate the median lethal dose and to examine the course of pathogenesis of EEEV disease. Such assessment is critical for the development of the marmoset as an animal model that can realistically mimic human disease and to compare the responses in the marmoset to other animal models of EEEV disease following aerosol infection (e.g., cynomolgus macaques). Some of the features of human disease which have been observed in the cynomolgus macaque model of EEEV infection include clinical and pathological changes (Reed, 2007). We describe the differential effects of dose on the immune cell populations in the blood, blood chemistry, viral burden in tissue, fever, body weight, and changes in blood oxygen saturation levels as part of the clinical symptoms and

pathology. These results, particularly the differential dose effects of aerosolized EEEV on fever, virus dissemination, neurological signs, and hematological parameters, reveal that the common marmoset is a promising model of lethal inhalational EEEV infection for use in the development of medical countermeasures.

Materials and Methods

Animals

Five healthy, adult common marmosets (*Callithrix jacchus*) (1 male, 4 females) were obtained from the United States Army Medical Research Institute of Infectious Diseases (USAMRIID) nonhuman primate colony. All marmosets were determined to be naïve to previous alphavirus infection and to be free of common opportunistic pathogens to include lymphocytic choriomeningitis virus (LCMV) *Salmonella*, *Shigella*, *Camplyobacter*, and *Klebsiella pneumoniae*. All marmosets ranged between 290 and 375 grams in weight and were between 2 and 4 years of age at the time of study. Marmosets were given water *ad libitum*, received the customary marmoset diet twice daily, dietary enrichment daily following exposure, as well as conventional environmental enrichment. Animals were surgically implanted with subcutaneous Data Sciences International (DSI) TA-F40 telemetry implants to remotely monitor temperature. Approximately one week prior to aerosol exposure, animals were moved to biosafety level-3 facilities at USAMRIID and housed in cages that were modified for marmosets. The animals were housed in rooms that were maintained at approximately 25°C and on a 12 hr light/dark cycle.

Research was conducted under an IACUC-approved protocol in compliance with the Animal Welfare Act, PHS Policy, and other Federal statutes and regulations relating to animals and experiments involving animals. The facility where this research was conducted is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011.

Virus

The FL93-939 strain is a prototype North American EEEV strain. It was originally isolated from a pool of mosquitoes (*Culiseta melanura)* from Florida in 1993. The virus was a kind gift from Dr. Scott Weaver, University of Texas Medical Branch. The virus isolate was subjected to one pass through C636 cells, one pass in suckling mouse brain, one pass on Vero cells, and one pass on BHK cells at USAMRIID to produce the sucrose-purified stock. Purified virus was diluted to the appropriate concentration in unsupplemented Eagle's Modified Essential Medium with non-essential amino acids (EMEM w/NEAA) prior to aerosol exposure.

Aerosol Challenge

In preparation for aerosol challenge, marmosets were initially anesthetized with Isoflurane and maintained with Ketamine-Acepromazine (Ket-Ace) during the aerosol exposure procedure. Each marmoset was exposed to aerosolized EEEV in a head-only exposure chamber contained in a class III biological safety cabinet inside a BSL-3 laboratory. Aerosol exposure was controlled and monitored using the Automated Bioaerosol Exposure system (ABES) (Hartings & Roy, 2004). Delivery of the target aerosol dose was based on calculations of minute volume based on Guyton's formula, taking into consideration: (1) the flow to volume ratio of the exposure chamber, (2) the starting EEEV concentration in the Collison nebulizer and (3) the spray factor calculated from sham experiments using the virus stock (Guyton, 1947). All exposures were generated with a three-jet Collision nebulizer and air passing through the exposure chamber was collected for sampling in an all-glass impinger (AGI) (Reed, 2004). Titer of the aerosolized agent collected in AGIs was determined for each exposure by viral plaque assay. The actual inhaled dose of EEEV was calculated based on the concentration and volume

of the AGI samples, the estimated minute volume, and flow rate of the aerosol sampler using the following formula:

$$\text{Inhaled Dose} = C(AGI) \times V(AGI) \times MV / Q(AGI)$$

Where inhaled dose (PFU) is calculated based on: C, the concentration (PFU/ml) of the virus sampled from the AGI; V, the volume contained in the AGI sample (ml); MV, the minute volume (ml/min) for each animal estimated from Guyton's formula; and Q, the flow rate of the AGI sampler (ml/min).

Telemetry Analysis

Prior to aerosol challenge, all marmosets were surgically implanted with a subcutaneous Data Sciences International (DSI) (St. Paul, MN) radiotelemetry device (TA-F40) to monitor body temperature and activity. Temperature was recorded using the DataQuest A.R.T 4.1 system (DSI). The system was set to collect data every five minutes, starting at 7 days prior to aerosol exposure and continuing until day 28 post-exposure or earlier if euthanasia criteria were met. Bayesian estimation of the distribution of daytime body temperature for each marmoset prior to aerosol challenge was used to compute a credible range for body temperatures using SAS Markov chain Monte Carlo simulation procedure (PROC MCMC). Data analysis included temperature measurements that were compatible with life (i.e., $\leq 42°C$). A 99.7% credible range was generated for each animal's daytime body temperature, analogous to an interval of ±3 standard deviations for a normally distributed variable. All post-aerosol challenge temperature readings were compared to the expected temperature interval estimated for each animal. Temperature measurements above the upper limit of the estimated interval were noted as elevated and used to compute fever summary statistics.

Observation and Clinical Evaluation

Marmoset clinical observations began three days prior to aerosol exposure for a baseline appearance and behavior appraisal and continued minimally twice daily post-exposure. Several factors were used when evaluating clinical signs of disease for each marmoset. Clinical observation parameters included: (1) neurological signs (0 = normal; 1 = loss of coordination; 2 = occasional tremors; 3 = loss of balance; 4 = frequent tremors/seizures), (2) temperature (0 =normal; 1 = > 1°C above baseline; 2 = 2°C above baseline; 3 = 3°C above or below baseline; 15 = 4°C below baseline), (3) appearance (0 = normal; 1 = reduced grooming; 2 = dull/ rough coat, ocular nasal discharge; 3 = lethargy; 4 = piloerection, hunched up), (4) natural behavior (0 = normal; 1 = minor changes, 2 = little peer interaction, less mobile, 3 = no peer interaction, vocalization, or self-mutilation) and (5) provoked behavior (0 = normal; 1 = subdued when not stimulated, 2 = subdued when stimulated, 15 = unresponsive/weak, pre-comatose). To further assess the health status of the marmosets, animals were anesthetized every three days for collection of weight and to conduct a physical examination. During this time, blood samples were collected for complete blood count (CBC) analysis. White blood cell (WBC) counts were included in the clinical scoring of the animals. Scoring criteria for WBC were as follows: 0 = normal; 1 = 10-12 K/ µl; 2 = 12-14 K/µl; 3 = 14-20 K/µl; 4 = > 20 K/µl. In addition, blood oxygen saturation was determined every three days using a pulse oximeter. Animals with a total clinical score $\geq$15 met euthanasia criteria.

Plaque Assay

Dissemination of infectious virus in blood and tissues was assessed by plaque assay. Briefly, USAMRIID Vero 76 cells were seeded on 6 well tissue culture plates and grown to 90-95% confluence. Samples were serially diluted in Hanks' Balanced Saline Solution (HBSS). Cells were infected with 0.1 mL of serially diluted samples per well, in triplicate. Plates were

incubated at 37°C, 5% CO_2 for 1h with gentle rocking every 15 minutes. After 1h, cells were overlaid with BME (Gibco A15950DK) containing 0.6 % agar supplemented with 10% heat-inactivated FBS, 2% Penicillin/Streptomycin (10,000 IU/ml and 10,000 µg/ml, respectively), and further incubated for 24h at 37°C, 5% CO_2. A second agarose overlay, prepared as described above, containing 5% neutral red vital stain (Gibco 02-0066DG) was added to wells and further incubated for 18-24h for visualization of plaques and determination of viral concentration in each sample [virus plaque forming units (PFU) value].

Semi-Quantitative RT-PCR

Viral RNA was detected by reverse-transcriptase polymerase chain reaction (RT-PCR). Viral RNA from blood and swab samples was isolated using QIAamp Viral RNA mini kit (QIAGEN, Valencia, CA). RNA from tissue samples was isolated using RNeasy mini-protect kit (QIAGEN, Valencia, CA). Semi-quantitative reverse transcriptase-PCR (RT-PCR) was used for detection of viral RNA from oral swab samples as well as blood and tissue samples from the study marmosets. The EEEV viral RNA assay was designed to amplify a portion of nonstructural protein 1 (nsp1) of the Georgia 97 strain of EEEV using the following primers and probe, respectively: EEEV Forward 5'-TGCAAAgATGCTTTCC-3', EEEV Reverse 5'-TCACCTGGTCTGTATCCA-3', and the dual-labeled TaqMan probe 5'-CAACGCAGGTCACTGACAAT-3'. Quantification of viral RNA in samples was achieved by comparison of unknown blood and tissue samples to an RNA standard generated from EEEV FL93-939 virus, which is the same virus strain used for the aerosol exposure of experimental animals. The standard curve ranged from 5.0×10^7 [upper limit of detection (ULOQ)] to 5.0×10^2 viral RNA copies [lower limit of detection (LLOQ)]. Repeated attempts to amplify virus below the LLOQ failed to consistently and reliably demonstrate amplification, thus the LLOQ

was set at 5.0×10^2 viral RNA copies. Positive and negative extraction controls were created by supplementing uninfected NHP blood with a known amount of EEEV FL93-939 virus (5.0×10^4 viral genomic copies) and RNase-free water, respectively.

Pathology

Necropsies were performed on each marmoset under BSL-3 conditions. Tissues were collected from all major organs in the body for histopathological and immunohistochemical assessment. Tissues were immersion fixed in 10% buffered formalin and held in biocontainment for a minimum of 21 days. Tissues for histopathology underwent routine histologic processing, were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Immunohistochemistry was performed on all tissue sections using a rabbit anti-Alphavirus (#1140) antibody (1:8000 dilution) and a commercial immunoperoxidase detection kit (EnVision System, Dako Corp., Carpinteria, CA). After pretreatment with a TRIS/EDTA buffer (pH 9.0) at 97°C for 30 minutes, primary and secondary antibodies were applied and the slides were incubated with substrate-chromagen solution according to the manufacturer's recommendations. Sections were counterstained with hematoxylin.

Results

Aerosol Exposure

To evaluate the common marmoset (*Callithrix jacchus*) as an animal model of aerosolized EEE, five marmosets were challenged with increasing doses of EEEV in a whole-body aerosol exposure chamber and then observed to determine how well the marmoset model emulated human disease. The individual, inhaled doses obtained for each of the exposed marmosets were: 2.40×10^1 PFU, 1.15×10^3 PFU, 1.20×10^4 PFU, 9.76×10^4 PFU and 7.95×10^5 PFU. At the doses of aerosolized EEEV tested, three marmosets reached euthanasia criteria, marmoset 5 (highest inhaled dose of 7.95×10^5 PFU) was found dead in the cage, and marmoset 1, which received the lowest dose (2.40×10^1 PFU), survived throughout the period of observation. Clinical and physiological changes were assessed and diagnostic tests were used to monitor blood and tissue dissemination of the virus in the marmosets.

Clinical signs

Marmosets were observed for the development of clinical signs indicative of EEE disease in accordance with the scoring system described in the Material and Methods section. Subtle changes in the appearance and behavior of animals was observed as early as day 2 following EEEV aerosol exposure, and by day 3, all marmosets had presented with clinical signs (Figure 1). The marmoset receiving the highest dose of aerosolized EEEV (Marmoset 5, inhaled dose 7.95×10^5 PFU) showed clinical signs as early as day 2 following exposure and was found dead in the cage on day 4. This marmoset succumbed to disease quickly at this dose and therefore displayed a much more abbreviated disease course. The next marmoset received an aerosol dose that was approximately under one half -log below the inhaled dose of marmoset 5 (marmoset 4, inhaled dose 9.76×10^4 PFU). Marmoset 4 displayed a similar, but more prolonged

course of disease than that of Marmoset 5. The clinical scores were higher between days 3 and 4 and Marmoset 4 reached euthanasia criteria by day 6, two days later than marmoset 5. The lowest lethal inhaled dose achieved was 1.15×10^3 PFU (marmoset 2), which resulted in a prolonged study observation period (marmoset reached euthanasia criteria on day 19 post exposure). Marmoset 3 received an intermediate dose of 1.2×10^4 PFU which corresponded to a survival profile observed to fall between the lower and higher doses; the marmoset reached euthanasia criteria on day 12 post exposure. Marmoset 1 (inhaled dose: 2.4×10^1 PFU) survived and displayed only marginal, observable clinical changes, mainly between days 4 and 12. Ruffled fur and reduced grooming of marmoset 1 were the first visual signs of infection to be noted in the daily observations. Lethargy, decreased interaction, slight piloerection, tremors, and lack of balance were noted in several of the animals that met euthanasia criteria and received higher inhaled doses of EEEV. At later times in the disease course, marmosets became more subdued in their behavior and response to stimulation was absent, even when provoked.

The effect of EEEV exposure on marmoset body weight was assessed. Considerable weight loss was observed between day -3 and study endpoint in marmosets 3 (inhaled dose 1.2×10^4 PFU) and marmoset 4 (inhaled dose 9.76×10^4 PFU) with a loss in body weight of 6.25% and 13.3%, respectively (Figure 2). The rapid progression of disease into lethality observed for Marmoset 5, which received the highest EEEV dose resulted in a marginal decrease in weight between day -3 and day 4 post exposure (endpoint). For doses below 1.2×10^4 PFU, a slight weight increase was observed (4.02% increase for marmoset 2) and 1.34% decrease for marmoset 1. These results indicate that weight loss was observed to be greater in marmosets that received higher aerosol doses. In fact, the dose range encompassing marmosets 3 and 4 was the critical interface where clinical manifestations and body weight loss were most evident.

We next addressed the hematological changes occurring in marmosets receiving distinct aerosol doses. For this purpose, CBCs were performed on samples collected pre-exposure and then on days 3, 6, 9, 12, 15, 18, 21, 24, 27 and at study endpoint (Figure 3). Marmosets that received lower doses of EEEV initially displayed a decrease in WBCs on days 3 to 9 post exposure (Figure 3A). Overall, an increase in total white blood cell (WBC) count was observed in marmosets that progressed through the disease course (Figure 3A). Sharp increases in WBCs and neutrophils (a clear contributor to the overall increase in WBCs) occurred during the final days of disease (Figures 3A and 3B, respectively) for marmosets that received a lethal dose. The increase in the number of lymphocytes (Figure 3C) followed a trend similar to that of the neutrophils (Figure 3B) for the marmosets receiving lethal aerosol doses. A biphasic lymphocyte response was noted for the surviving marmoset 1. A steady increase in monocytes was noted in marmosets 2 and 3 over the course of infection. There was a sharp increase in monocytes in marmoset 2between days 10-18. Likewise, monocytosis was observed for marmoset 1 (the lone surviving animal) between days 9 and 18, but resolved thereafter until the end of study (Figure 3D). In general, the number of platelets showed a downward trend over the course of infection. The surviving marmoset 1 had a substantial drop in platelets between days 3 and 9; however this was resolved by the end of the study (Figure 3E). No animal displayed signs of anemia during the course of study; levels for hemoglobin, hematocrit, and RBC remained within normal levels throughout the study (data not shown).

Fever onset and duration, followed a dose-dependent pattern (Figure 4). With the exception of marmoset 1 that received the lowest dose, all other marmosets developed fever as defined by a temperature change that was three standard deviations (SD) over the average body temperature for that animal and occurring on at least three consecutive readings (Figure 4 and

Table 1). Although marmoset 1 revealed sporadic spikes in temperature resembling fever, those spikes did not occur continuously (data not shown). This animal was the only one which survived aerosol exposure until the end of the study. In marmosets receiving the highest EEEV doses (marmosets 4 and 5), fever occurred earlier and had a shorter duration (see Figures 1, 4D, 4E, and Table 1), and for lower inhaled doses, fever was more prolonged as was the course of the disease (see Figures 1, 4B, 4C, and Table 1). The common aspect for the marmosets that met euthanasia criteria was an increase in body temperature (fever), at times considerable, immediately followed by a noticeable drop in the animal's body temperature as the animal became moribund (Figure 4B-E). In contrast, the surviving marmoset (marmoset 1) retained a steady body temperature throughout the course of the study without onset of fever. In the exposed marmosets, fever persisted for 25 hr to 122 hr. Marmoset 2, which received the lowest lethal aerosol dose of EEEV, exhibited the most prolonged period of fever (Table 1). In all animals, fever peaked at approximately 40^{o}C, except for marmoset 5 which peaked at 43.6^{o}C.

Oxygen saturation levels in blood were also collected to further evaluate the health status of the marmosets in the study. These data were obtained using a pulse oximeter (Figure 5). Variation in oxygen saturation was more evident in marmosets receiving intermediate doses of EEEV aerosol (i.e., marmoset 3: 1.20 x 10^4 PFU and marmoset 4: 9.76 x 10^4 PFU in Figure 5. The sharp change in oxygen saturation observed for the marmosets exposed to intermediate doses were not noted in those animals receiving either the highest or the two lowest EEEV doses. No dramatic changes in percent oxygen saturation were noted in marmosets that received highest and two lower doses of EEEV. Compared to pre-exposure values (day -3), percent oxygen saturation decreased from 94% at day -3 pre-aerosol exposure to 86% at study endpoint (day

+12) for marmoset 3 (Figure 5). A similar drop was seen for marmoset 4 (9.76×10^4 PFU) from 98% (day -3) to 87% (study endpoint, day +6).

Viral Dissemination

The presence of virus in tissues, whole blood, and oral swabs collected from infected marmosets was assessed either by plaque assay or by RT-PCR. Tissues were collected from the marmosets at necropsy, while the blood and oral swab samples were collected 3 days before aerosol exposure and compared to samples taken at least every 3 days after aerosol exposure at physical examination while under anesthesia. Infection was found to be widely disseminated in tissues of marmosets that received the higher EEEV aerosol doses (see marmosets 3, 4 and 5 in Table 2). Marmoset 1 which received a non-lethal dose was the only animal with no detectable EEEV in tissues by either plaque assay or RT-PCR (Table 2). Virus was detected in brain by at least one of the detection methods in all marmosets that received a lethal dose (i.e., marmosets 2 through 5). The liver and kidney had detectable levels (by at least one method) of virus in the marmosets that received the three highest EEEV doses (i.e., marmosets 3 through 5). EEEV was detected in the mesenteric lymph node and heart of marmosets receiving high doses of aerosolized EEEV. Virus was detected in the lungs only by plaque assay and only in the marmoset that received the highest dose of EEEV.

EEEV was detected in the inguinal and mandibular lymph nodes by both plaque assay and RT-PCR in marmoset 3, an animal that received an intermediate dose of EEEV and survived to day 12 post exposure. The adrenal gland was positive in the marmoset that received the highest dose. Only marmoset 1 tested positive for EEEV RNA in the blood by RT-PCR at days 9 and 12 post (Table 3). When assessed by plaque assay, viremia was not detected in any animals at any time point. Oral swabs were also negative for EEEV by either plaque assay or RT-PCR.

Comparison of results between plaque assay and RT-PCR should be approached with caution as differences in sensitivity and sample recovery between these two assays may help explain, at least in part, why some samples are positive for one assay but not the other.

Pathology

No gross lesions were present at necropsy in any animal, irrespective of dose or disease outcome. Histologic evaluation revealed few changes between doses in the animals that reached euthanasia criteria. Clinical disease progressed similarly, but the observations varied temporally depending on the aerosol dose with the exception of the observation of vasculitis. Vasculitis was apparent in marmosets that received doses greater than 1.0×10^4 PFU. The histologic changes included acute to subacute meningoencephalitis (Figure 6A) with neuronal necrosis and prominent vasculitis (Figure 6B) present within the brain of marmosets infected at higher doses ($>1.0 \times 10^4$ PFU). The surviving marmoset, however, did not have any histologic changes present. Of those with histologic lesions, the portions of the brain most severely affected were the frontal cortex, corpus striatum, thalamus; mesencephalon, pons, medulla oblongata and cerebellum. The meningoencephalitis consisted of infiltration of mononuclear inflammatory cells with high numbers of neutrophils. Additional changes described included neuronal cell death (Figure 6C and 6D), gliosis, satellitosis, edema, and vasculitis. Hemorrhage was occasionally present. Table 4 summarizes the pathologies observed in the EEEV infected marmosets.

Presence of EEEV in select tissues was demonstrated using immunohistochemistry (IHC). Positive IHC staining for EEEV antigen was observed in neurons within the brain (Figure 7) and the retina. Regions of brain with strongest IHC labeling were the frontal cortex, corpus striatum, thalamus, mesencephalon, and pons. Other tissues with positive IHC staning included

359 macrophages/dendritic cells within the mandibular and axillary lymph nodes, interstitial cells of
360 the ovary, and cells of the inner ear (data not shown). Nasal turbinates, nasal septum, and tooth
361 pulp were also examined but were negative for the presence of viral antigen by IHC staining. No
362 histologic changes were noted in the retina, despite positive IHC antigen staining (Tables 4 and
363 5).

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Discussion

New world alphaviruses represent a recognized biological threat that can be intentionally delivered by aerosol; new vaccines are being developed but must be tested for efficacy against alphavirus inhalation exposure (Spurgers & Glass, 2011). Currently, efficacy testing relies greatly on the availability of adequate animal models which can emulate human disease. The overarching purpose of the present work was to investigate the common marmoset (*Callithrix jacchus*) as a model of inhalational disease for EEEV.

In the past, the mouse has been routinely used as an animal model, but because mice can develop natural immunity to peripheral EEEV challenge, the use of this model has to be carefully considered (Steele & Twenhafel, 2010). In addition to mice, various other animal models have also been used in inhalation studies of EEEV, including hamsters, guinea pigs, and both rhesus and cynomolgus macaque non-human primates (NHP). Although mice and hamster are highly sensitive inhalation models of alphavirus disease, the vascular manifestation that is usually fatal in humans is a shortfall for the murine model (Lui, 1970) and the fulminant course of disease is a limitation in the hamster (Rebecca Erwin-Cohen, personal communication). Guinea pigs demonstrate a disease profile consistent with that observed in humans including the development of neurological sequelae in the surviving animals (Roy, 2009; Rebecca Erwin-Cohen, personal communication). The effects of EEEV infection have been assessed in both rhesus and cynomolgus macaque nonhuman primate species (Dupuy & Reed, 2012) and in equine models (Hays, 1969). Some of these previous studies have focused on the aerosol route of exposure in nonhuman primates (Reed, 2005; Reed, 2007; Dupuy, 2010; Dupuy & Reed, 2012). The report on the common marmoset comparing virulence of intranasal infections between North American (NA) and South American (SA) strains of EEEV (re-classified as Madariaga Virus) was

promising as comparable responses to humans were found in the marmoset model (Aguilar 2005; Adams, 2008). The fact that in this study marmosets developed vasculitis and have previously been reported to produce neutralizing antibodies following infections with low virulence alphavirus strains was interpreted as a promising indication of this model as being suited for inhalational studies. Collectively, these factors prompted us to evaluate the marmoset for its suitability as a model of EEEV disease following aerosol route.

To determine the effect of the aerosol dose on the infectivity and disease development for the marmoset, inhaled doses ranging from 2.4×10^{1}PFU to 7.95×10^{5} PFU were tested. Markers of EEEV infection and disease were observed. As the dose increased, so too did the severity of the clinical disease course; marmosets reached euthanasia criteria progressively earlier and displayed a more abbreviated list of clinical signs and symptoms, revealing the dose-dependent nature of EEE manifestation. A gradient of disease severity was observed within the lethal doses. For example, marmosets receiving the two highest doses of EEEV succumbed to disease by days 4 and 6, respectively. This dose range revealed a similar effect as that observed when marmosets were exposed by the intranasal route (i.n.) with 1.0×10^{6} PFU of the EEEV FL93-939 strain (Adams, 2008). In Adams' work, the time of death and the host responses observed were similar to what we observed in the present study. However, in our study, marmosets exposed to lower doses of EEEV displayed a protracted time to death, and the surviving marmoset exposed to the lowest aerosol dose remained nearly asymptomatic throughout the course of the study. The responses of the surviving marmoset more closely resembled those of marmosets exposed i.n. to the SA strain of EEEV (Madariaga Virus) in Adams' work; suggesting a dose threshold for NA strain toxicity that responds similarly to high i.n. doses of the less virulent SA strain (Madariaga Virus).

Loss of body weight was demonstrated to be a factor in the disease pathology of intranasal EEEV infection in marmosets and in the human disease (Adams, 2008). In our study, loss of body weight was observed between day -3 and study endpoint within a dose range of aerosolized EEEV (i.e., between 1.2×10^4 PFU and 9.76×10^4 PFU). Doses below this range either had no effect or the marmoset gained weight, regardless of whether the animal developed disease leading to euthanasia or not, indicating that these lower doses were too weak to allow for observable weight loss. Adams (2008) detected weight loss in all marmosets exposed i.n. with the EEEV FL93-939 strain at 1.0×10^6 PFU. Although we did not observe a significant change in body weight between pre-exposure values and study endpoint for a marmoset exposed to a similar dose of the NA strain, that same marmoset had a 4.2% decrease in body weight between day 3 and day 4 (study endpoint) post exposure. When marmosets were inoculated i.n. with an SA strain (Madariaga Virus) in Adams' study, they gained weight (Adams et al., 2008). Challenge with the SA strain of EEEV in that same study resembled the results we have seen for the marmoset receiving a lower dose of NA EEEV strain (i.e., 1.15×10^3 PFU). Because we covered a wider dose range of EEEV doses in our median lethal dose study and possibly due to the use of a different route of exposure, we were able to capture differential effects on weight not previously observed before for the same EEEV strain (Adams, 2008).

We also noted that marmosets exposed to the higher doses of EEEV ($\geq 9.76 \times 10^4$ PFU) developed disease and reached euthanasia criteria at roughly 2-3 days following the time of fever onset. This observation is in agreement with Adams' work as well as with previous data from cynomolgus macaques (Reed, 2007; Adams, 2008). Rhesus macaques have also been exposed to EEEV but displayed a more extended survival time following fever onset (Wickoff & Tesar, 1939). The delayed effect on time to death in the rhesus macaque model was also observed in our

study, but only for marmosets which were exposed to the lower EEEV doses. These differences in the more prolonged time to death from fever onset for the rhesus may represent a species difference or be a reflection of distinct properties of the viral strains used as well as conditions of viral stock preparation and stability (Wickoff & Tesar, 1939). In the intermediate inhaled doses of EEEV in the marmosets, it was noticed that there was an increase in nocturnal activity for marmosets 2 at day 6 and for marmoset 4 at day 5 (data not shown); again, showing that within the "intermediate" range of inhaled doses, a more clearly traceable pattern of EEEV-driven effects could be detected. Interestingly, effects on blood oxygen saturation were more noticeable in this intermediate range, where weight loss was also considerably affected. A decrease in oxygen saturation levels in the blood has been previously shown in human influenza cases to reflect ongoing inflammatory responses and those results correlated with body weight loss, body temperature changes, and development of lung pathology (Verhoeven, 2009). The observed depression in oxygen saturation levels in the marmosets may also signal inflammatory responses captured in the animals within these intermediate EEEV aerosol doses.

In this study, pathological evaluation revealed few changes between animals at varying inhaled doses, with regard to the animals that had clinical disease and reached euthanasia criteria. To clarify, although the disease progressed on an altered time course dependent on the aerosol dose, once animals developed clinical disease, the pathological changes were similar with the exception of vasculitis. Vasculitis was present in doses greater than 1.0×10^4 PFU, suggesting it may a dose-related occurrence. Vasculitis is a pathology feature of human EEEV disease and emphasizes the importance of not only providing an animal model that will develop vasculitis, but provides insight into the aerosol dose required to mimic the disease course in humans. Therefore, aerosol doses greater than 1.0×10^4 PFU should be considered when using the

457 marmoset as a disease model for aerosolized EEEV. Meningoencephalitis was also noted in our
458 marmosets, which is consistent with observations made for fatal human EEEV cases (Feemster,
459 1938). We observed positive antigen staining is the pons and thalamus regions of the brain from
460 several of the marmosets; this observation of involvement of specific areas of the brain is
461 consistent with reports of EEEV-induced signaling anomalies in the basal ganglia, brainstem,
462 and thalamus from Magnetic Resonance Imaging (MRI) in both severe non-fatal and fatal human
463 infections (Min, 2013; Babi, 2014; Baig, 2014).

464 Viremia was not detected in the blood by plaque assay at any time point, but viral RNA
465 was detected by RT-PCR at days 9 and 12 post aerosol exposure in the survivor marmoset
466 receiving the lowest dose. The presence of EEEV RNA in the blood suggests a possible dose-
467 dependent or virulence-dependent, transient change in the dissemination pattern of the virus or
468 may simply reveal unique animal-specific responses. Indeed, the lack of either infectious EEEV
469 or viral RNA in whole blood at early time points may be a reflection of limited availability of
470 leukocytes within the whole blood samples at those particular times. Expression of EEEV has
471 been shown to be restricted to myeloid cells due to binding of microRNA miR-142-3p at three
472 conserved target sites within the 3'UTR of the EEEV genome, which serves to functionally
473 block viral replication in cell types other than myeloid cells (Guo 2014; Trobaugh, 2014).
474 Overall, our data are in agreement with previous work in marmosets that showed no viremia
475 developed at a high dose with the NA strain of EEEV but viremia was detected when the
476 marmosets were infected i.n. with the SA strain (now re-classified as Madariaga Virus) (Adams
477 et al., 2008). It is possible that the cell-type constraints found in EEEV due to binding of
478 miR142-3p in a conserved region of the 3' UTR do not occur in Madariaga Virus.

The main hematological change previously reported for EEEV infection is leukocytosis late in infection, reflecting in great part the induction of granulocytes (Reed, 2007; Adams, 2008). Not only were general increases in white blood cells observed but also increases in absolute numbers of neutrophils, lymphocytes and monocytes. Interestingly, monocytes have been shown to be refractory to EEEV infection, (Liprandi, 1986; Gardner, 2008), with a possible exception of an immortalized monocytic cell line pre-treated with a mitogen (Levitt, 1979). Outside of the known resistance of monocytes to EEEV infection, these cells, along with lymphocytes, were induced by EEEV in our work, even at non-lethal aerosol doses, underscoring their role in modulating EEEV infection. In addition, a downward trend in the number of platelets was observed after EEEV exposure, although it is not as conclusive for the survivor. A decrease in platelet numbers is found in infected humans as well (Harvala, 2009). Platelet depletion has been previously associated with the increasing development of vasculitis and potential coagulopathies. The hematology data from the marmosets therefore describes an early initiated neutrophilic response followed by a lymphocytic response in the acute phase and culminating with monocytosis; this observation was more evident in marmosets receiving lower doses of the virus. Because high dose animals did not live long enough for the body to fully respond with monocytosis, this appears to be a dose related event, where lower doses may allow the animal to live long enough to mount a fully-fledged immune response, which includes monocytosis.

Limitations to the study include the small number of animals tested to estimate the median lethal dose; however, the clinical, hematologic, and pathology data are consistent with reports from other nonhuman models of infection and thus support the use of the marmoset as a novel aerosol model of inhalation EEE for countermeasure efficacy testing. Follow on studies

will build upon the work described herein to further refine the median lethal dose with larger numbers of animals to achieve statistical significance, as well as investigate the temporal changes in disease pathology.

Conclusion

In conclusion, this work has evaluated the clinical and pathological effects of aerosolized EEEV in the common marmoset and estimated the median lethal dose for aerosolized EEEV. The marmoset has now been shown to be a promising animal model for both the intranasal and aerosol routes of alphaviral encephalitic disease. We have demonstrated the pathological effects of EEEV disease over a range of doses, and describe disease markers that can used with the model for both therapeutic and prophylactic studies. The results demonstrate that the marmoset is an animal model suitable for emulation of human EEEV disease in the development of medical countermeasures.

Disclaimer

Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

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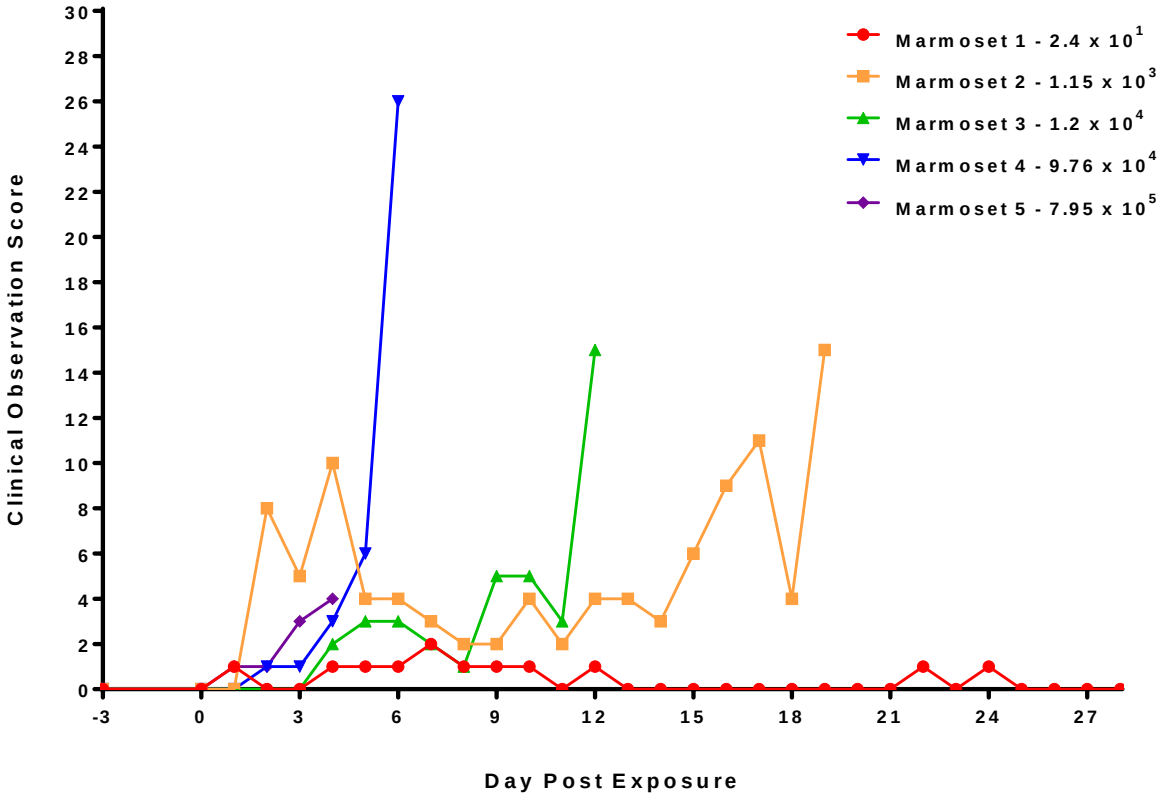
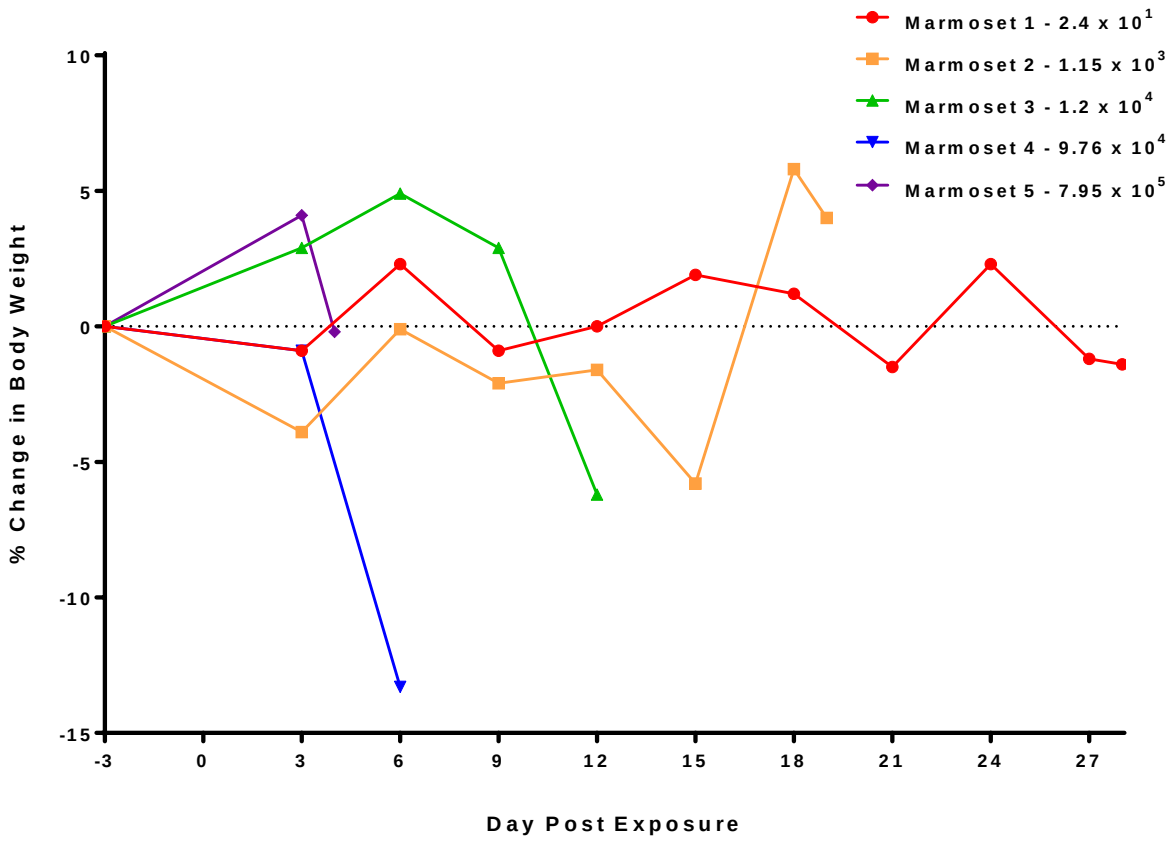


Figure 1. Clinical observation scores in marmosets with increasing doses of aerosolized EEEV. Clinical observation parameters included: (1) neurological signs, (2) temperature, (3) appearance, (4) natural behavior, and (5) provoked behavior. Animal behavior was noted and the sum of the score for each parameter was calculated. The values correspond to the highest score obtained for a marmoset per day.



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673 **Figure 2.** Effect of aerosolized EEEV on total body weight of marmosets. Marmoset weights
674 were recorded every third day during anesthetized physical observation. Weight loss was most
675 evident in Marmosets 3 and 4. The disease course, including weight loss, for Marmoset 2
676 followed a more prolonged course, consistent with the lower inhaled dose of EEEV that the
677 animal received. Marmoset 5 failed to show a meaningful decrease in body weight; however,
678 that animal was found dead on day 4 post exposure.

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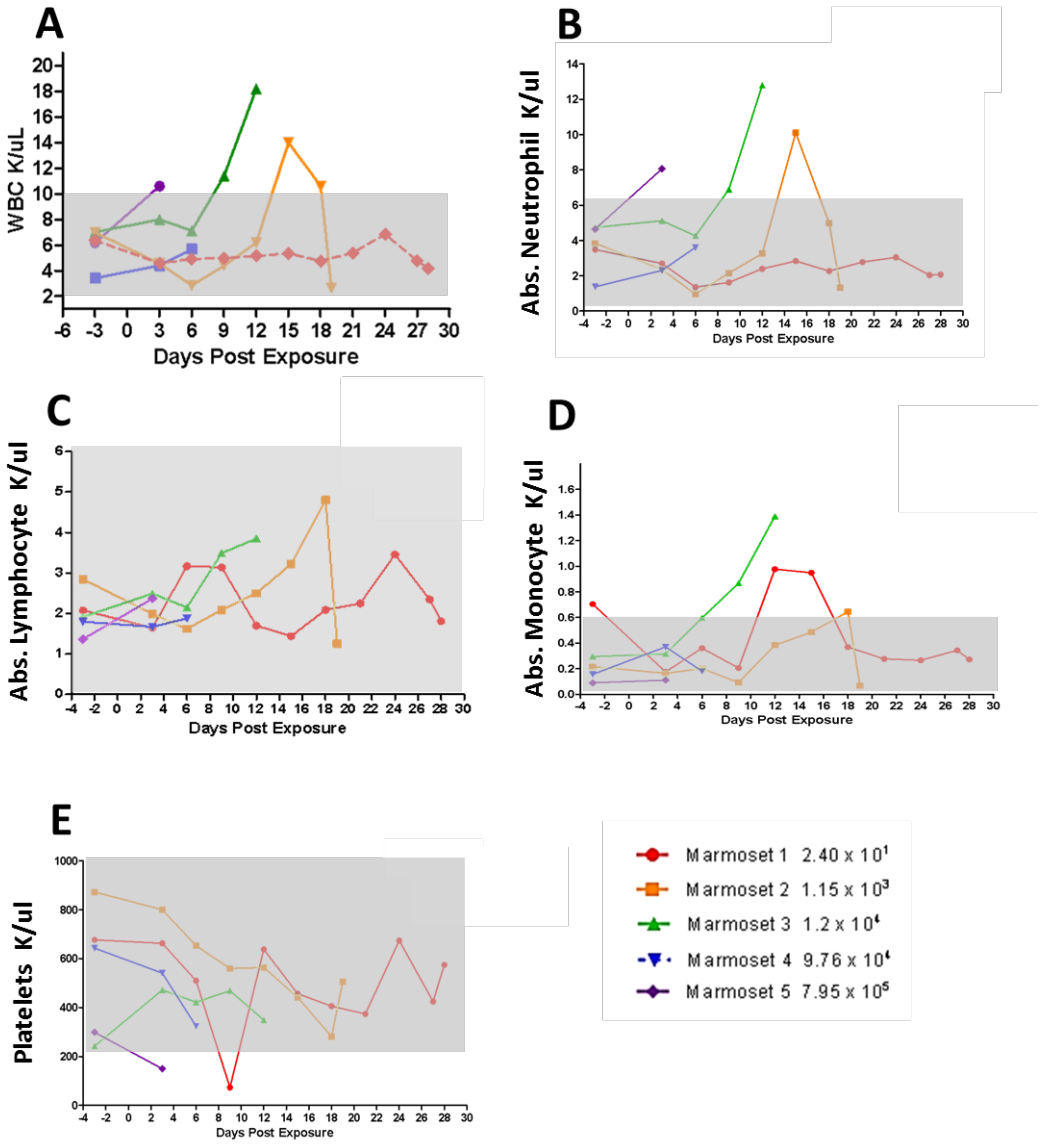


680

681 **Figure 3.** Hematological changes in marmosets after aerosolized EEEV challenge. The doses of
682 EEEV to which the marmosets were exposed are indicated in the bottom right of the figure. Grey
683 areas over the graphs correspond to normal value ranges for marmosets. The graphs show the
684 results for (A) white blood cell counts, (B) neutrophils, (C) lymphocytes, (D) monocytes and (E)
685 platelets in the infected marmosets through time.

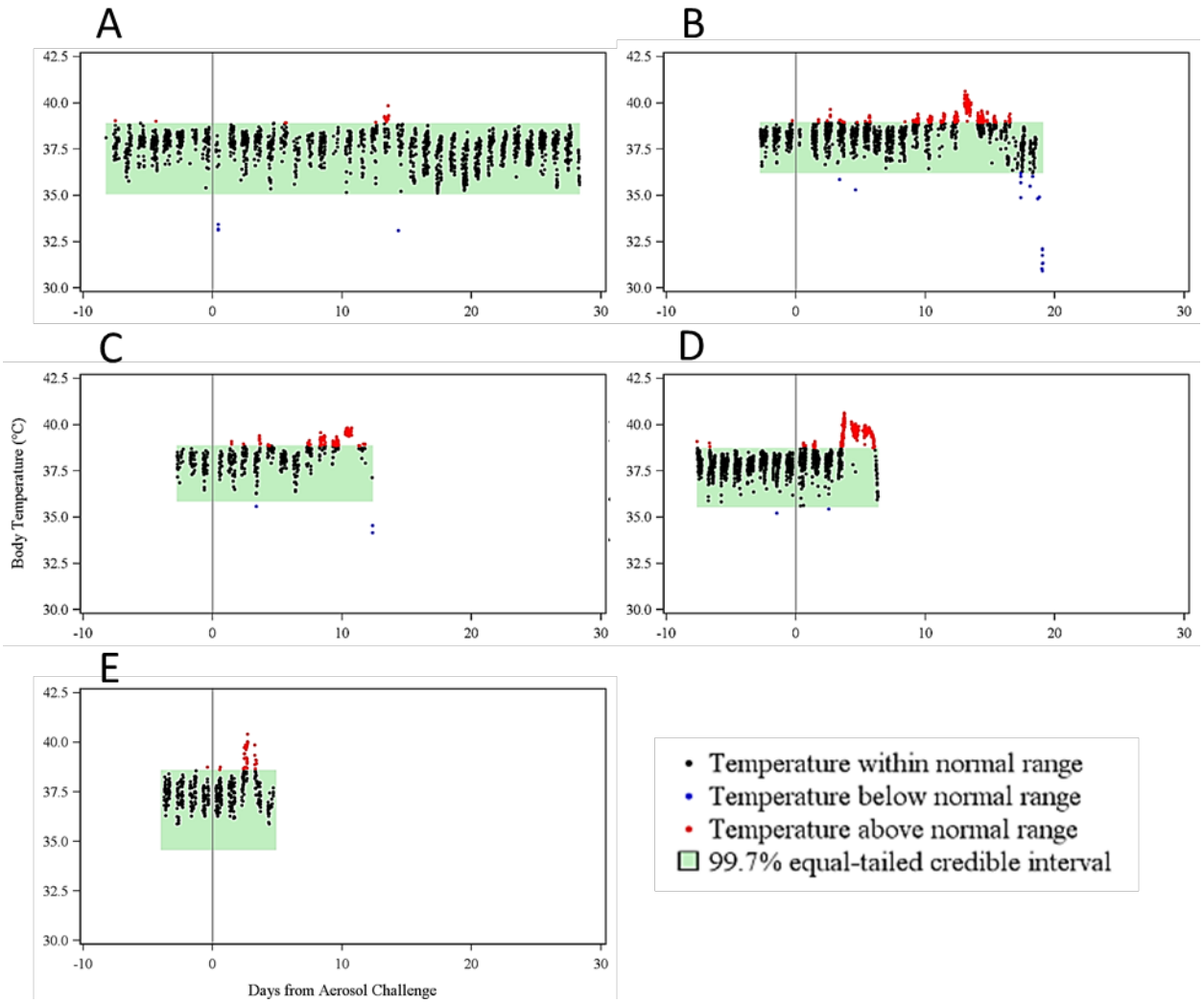
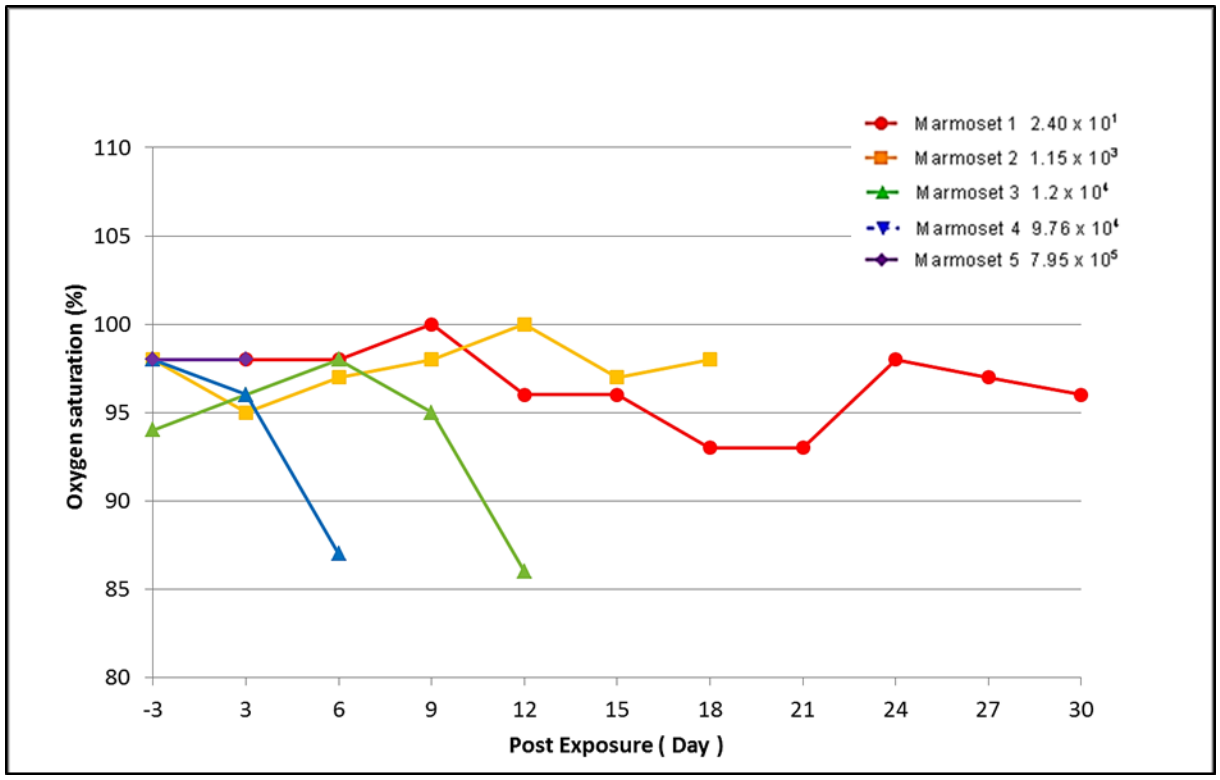


Figure 4. Fever response in marmosets after EEEV aerosol challenge. Variation in body temperature is shown for (A) Marmoset 1, (B) Marmoset 2, (C) Marmoset 3, (D) Marmoset 4 and (E) Marmoset 5. Fever was determined by comparing baseline body temperatures of the marmosets with temperatures measured after aerosol exposure. Baseline temperatures were collected every five minutes from as early as 7 days before challenge. Telemetry collection continued after exposure until study endpoint (up to 28 days post exposure). Average daily elevations in body temperature and any residual temperature data above 3 SD were used to compute fever duration.



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698 **Figure 5.** Changes in blood oxygen saturation in the marmosets was measured prior to and

699 following exposure to aerosolized EEEV. Blood oxygen saturation values were determined using

700 a pulse oximeter every 3 days while animals were under anesthesia for physical examination.

701

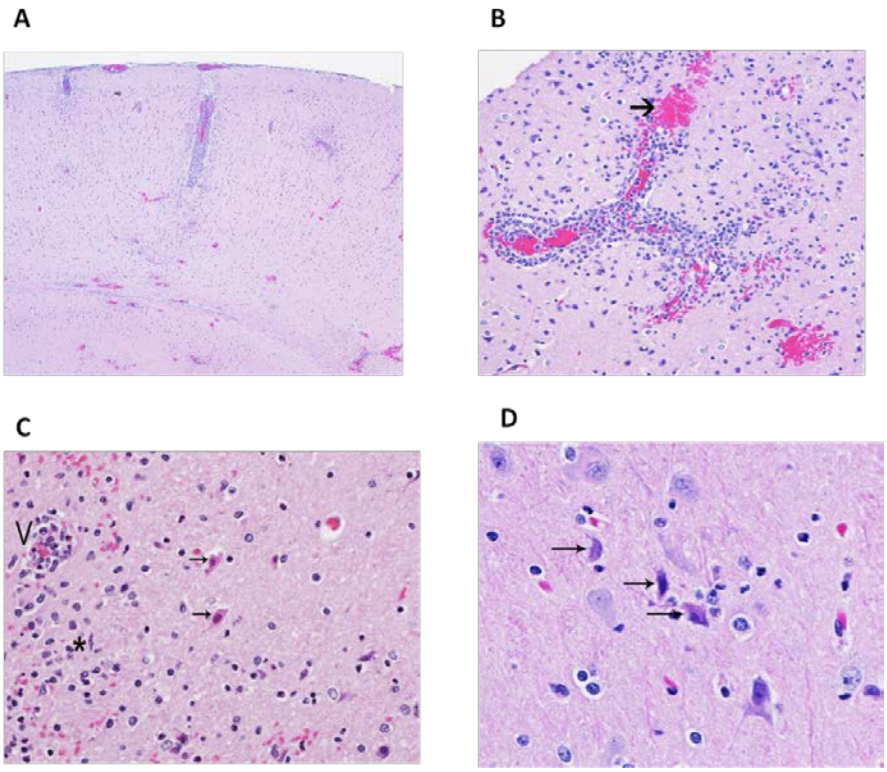

Figure 6. Histopathology following exposure to aerosolized EEEV. (A) Examination of the frontal cortex of the brain revealed the presence of multifocal meningoencephalitis with hemorrhage (HE; magnification, 4X). (B) Blood vessel displayed vasculitis with perivascular hemorrhage (arrow) (HE; magnification, 20X). (C) In the corpus striatum, two neurons (arrows) showed hypereosinophilic perikaryon (cytoplasm) suggesting necrosis. Vasculitis (V) with perivascular hemorrhage was seen in an adjacent vessel and gliosis (asterisk) in the surrounding neuropil (HE; magnification, 40X). (D) In the pons, three centrally located neurons (arrows) were observed that were shrunken and angular with hypereosinophilic perikaryon and deeply basophilic (hyperchromatic) nuclei (HE; magnification, 60X). Images are from marmoset 4 exposed to 9.76 x 10^4 PFU of EEEV.

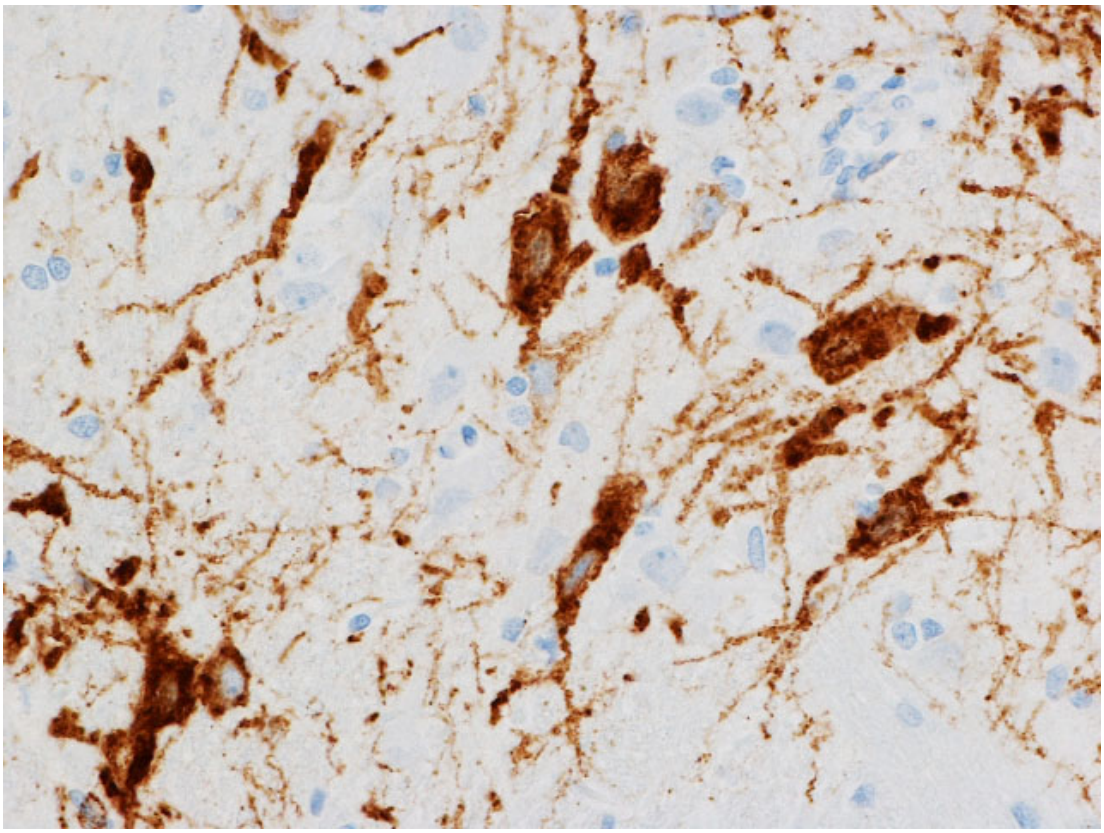

Figure 7. Immunohistochemistry analysis for marmosets exposed to aerosolized EEEV. This image from the pons reveals widespread positive staining of neurons in the brain, indicating the presence of EEEV (immunohistochemistry; magnification, 40X). Image is from marmoset 4 exposed to 9.76×10^4 PFU of EEEV.

721 **Table 1.** Summary of febrile responses in marmosets exposed to increasing doses of aerosolized
722 EEEV

Animal ID	Inhaled Dose (PFU)	Fever onset (day)	Duration (hr)	Fever Peak Temp (°C)	Last Temp Taken (°C)	Time of Death (day post exposure)
1	2.40×10^1	N/A	0	N/A	36.0	28
2	1.15×10^3	10	122	40.1	31.4	19
3	1.2×10^4	8	64	39.8	34.2	12
4	9.76×10^4	3	53	40.6	36.1	6
5	7.95×10^5	2	25	43.6	37.5	4

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725 **Table 2.** Comparison of tissue plaque assay (PA) and RT-PCR results from marmosets exposed
726 to aerosolized EEEV.

Tissue	Animal ID (Inhaled Dose, PFU)				
	1 (2.40×10^1)	2 (1.15×10^3)	3 (1.2×10^4)	4 (9.76×10^4)	5 (7.95×10^5)
Salivary Gland	-	-	-	-	-
Adrenal Gland	-	-	-	-	+
Pancreas	-	-	-	-	-
Lung	-	-	-	-	-
Spleen	-	-	+	+	-
Axillary LN	-	-	-	+	-
Kidney	-	-	+	+	-
Brain	-	+	+	+	+
Heart	-	-	-	-	+
Liver	-	-	+	-	+
Inguinal LN	-	-	-	-	-
Mandibular LN	-	-	+	-	-
Trachbronical LN	ND	-	+	-	+
Mesentric LN	-	-	-	-	+
Popliteal LN	-	-	+	-	-

ND =Not done
727 + = positive PCR result; - = negative PCR result

728 **Table 3.** Detection of EEEV in blood from infected marmosets by RT-PCR.

Animal ID (Inhaled Dose, PFU)	Sample Collection Day												
	-3	3	4	6	9	12	15	17	18	19	21	24	27
1 (2.40 x 10^1)	-	-	-	-	+	+	-	-	-	-	-	-	-
2 (1.15 x 10^3)	-	-	ND	-	-	-	-	-	-	-			
3 (1.20 x 10^4)	-	-	ND	-	-	-							
4 (9.76 x 10^4)	-	-	ND	-									
5 (7.95 x 10^5)	-	-	ND										

ND =Not done

729 + = positive PCR result; - = negative PCR result

730

731 **Table 4.** Observed Pathologies in marmosets challenged with EEEV by the aerosol route.

Marmoset	Inhaled Dose (PFU)	Time to Death Postexposure	Brain		Retina	
			Meningoencephalitis	Vasculitis	Retinitis	Vasculitis
1	2.40 x 10^1	28*	-	-	-	-
2	1.15 x 10^3	19	++	-	-	-
3	1.2 x 10^4	12	+++	+++	-	-
4	9.76 x 10^4	6	++	++	-	-
5	7.95 x 10^5	4**	+++	+++	-	-

* = Survivor, ** = Found dead

732 + = mildly present, ++ = moderately present, +++ = strongly/markedly present

733

734 **Table 5.** EEEV Immunohistochemistry Results for Marmosets challenged with EEEV by the

735 aerosol route.

Marmoset	Inhaled Dose (PFU)	Time to Death Postexposure	Brain	Retina
1	2.40×10^1	28*	-	-
2	1.15×10^3	19	+	-
3	1.2×10^4	12	+++	+
4	9.76×10^4	6	+++	+++
5	7.95×10^5	4**	+++	-

* = Survivor, ** = Found dead

736 + = mildly present, ++ = moderately present, +++ = strongly/markedly present

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